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INCREASED SEVERITY OF MURINE INFECTION WITH *TOXOPLASMA GONDII* FOLLOWING VITAMIN E AND SELENIUM SUPPLEMENTATION

A Thesis

Presented to the Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Susan McCarthy

December 1999

INCREASED SEVERITY OF MURINE INFECTION WITH *TOXOPLASMA GONDII* FOLLOWING VITAMIN E AND SELENIUM SUPPLEMENTATION

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ACKNOWLEDGEMENTS

I would like to extend a heartfelt thank you to Dr. Cheryl Davis for her continuous facilitation, support, and excellent direction throughout my entire thesis experience. Dr. Davis' mentorship and personal example always helped me strive and push myself to the next level.

I would also like to thank Dr. Doug McElroy for his superb advice and assistance with my statistical analysis, and Dr. Sigrid Jacobshagen for her special attention and interest in my work. I truly appreciate Dr. John Andersland for his enormous help during my research.

A special note of gratitude to Caroline (Flip) Nolen and her staff at Vanderbilt University for their assistance in the histological portion of my research.

I would also like to thank the Graduate Student Research Committee for funding my research, and the entire faculty in the Department of Biology for their support.

Last, I would like to thank my family, sister-in law, and best friend, Sherryl Lockett, for their encouragement, support and understanding during my research.

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Susan McCarthy December 1999 58 Pages

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At present, toxoplasmosis is one of the most common opportunistic infections in immunocompromised patients. The need for a reliable experimental model is crucial not only for achieving a better understanding of the pathophysiology of this infection but also for developing a better method to evaluate new therapeutic regimens. This study was organized to determine if the antioxidants vitamin E and selenium would provide a beneficial effect in mice chronically infected with *Toxoplasma gondii.* In the first phase of the study, 35 female Swiss Webster mice were infected with oocysts of the Me49 strain of *Toxoplasma gondii* while receiving diets supplemented with vitamin E alone or in combination with selenium, or a diet deficient in both nutrients. In the second phase of the study, 25 C57BL/6J mice were infected intraperitoneally (i.p) using the ME49 strain. Two different strains of mice were used for this experiment because each vary in their susceptibility to *T. gondii* infection, with the C57BL/6J mice being a more susceptible model with the development of toxoplasmic encephalitis. In the third phase of the study, because the natural route of infection is the oral route, 25 C57BL/6J mice were infected orally with the ME49 strain of *Toxoplasma.* The results of all three experiments demonstrate that vitamin E and Se supplementation does not provide a protective effect during murine *Toxoplasma gondii* infection. Mice fed diets supplemented with vitamin E and Se had more tissue cysts present in their brains, exhibited greater tissue pathology,

and suffered the highest percent weight loss. In contrast, the unsupplemented groups (absence of vitamin E and Se from the diet) showed the lowest tissue cyst numbers, minor histopathology, and very little weight loss during experimental infection.

BACKGROUND

Toxoplasma gondii

Toxoplasma gondi is the protozoan parasite responsible for toxoplasmosis in humans and other warm-blooded animals (Bourguin et al., 1993). This parasite can infect most warm-blooded animals, both domestic and wild, and thus is among the most cosmopolitan of parasites (Plorde, 1990). Infections by *T. gondii* are widely prevalent in humans throughout the world; approximately one-half of the human population of the United States has been infected (Dubey et al., 1996). However, in the overwhelming majority of cases the infection is chronic, asymptomatic, and self-limiting. Although generally benign for healthy people, toxoplasmosis may cause abortion or neonatal malformations if contracted during pregnancy. Furthermore, this disease is often lethal for immunocompromised patients such as those with AIDS, those with neoplastic disease, or bone marrow or heart transplant recipients (Bourguin et al., 1993). At least 30% of patients with AIDS who have antibodies to *T. gondii* will develop toxoplasmic encephalitis (Suzuki and Joh, 1994). Toxoplasmic encephalitis has emerged as a major cause of morbidity and mortality in patients with acquired immunodeficiency syndrome (AIDS) and is the most common cause of brain lesions (Suzuki et al., 1994). In veterinary medicine, toxoplasmosis has great economic importance in many parts of the world due to abortion in cattle and neonatal loss. Sheep seem to be particularly susceptible, and *Toxoplasma* –caused abortions in this host often reach epidemic proportions (Roberts and Janovy, 1996). Thus, the importance of the organism as a

human and animal pathogen has stimulated a significant amount of research in recent years.

Biology of the Parasite and Modes of Transmission

Toxoplasma gondii is an intracellular parasite within many kinds of tissues, including muscle and intestinal epithelium. It may inhabit the nucleus of the host cell but usually lives in the cytoplasm (Roberts and Janovy, 1996). This invasive parasite enters host cells and multiplies within a highly modified vacuole, which does not acidify or fuse with the host lysosomal system (Shaw et al., 1998). Although susceptible to oxidative killing, *T. gondii* efficiently enters the host cell (ie., macrophages) without triggering the production of hydrogen peroxides (H_2O_2) or other toxic O_2 metabolites. During and after entry, the parasite discharges its secretory organelles (rhopteries and dense granules), resulting in the accumulation of one or more parasite-derived proteins in the newly formed parasitophorous vacuole space (Joiner et al., 1990).

Sexual reproduction of *Toxoplasma gondii* occurs only in the definitive host, the felines, and most importantly the domestic cat. When a cat is infected with *T. gondii* the parasites enter the cells lining the cat's small intestine and undergo rapid asexual reproduction (Plorde, 1990). The parasites enter the epithelial cells of the ileum, and once inside an epithelial cell, the parasite becomes a trophozoite that grows and prepares for merogony. With cell rupture, merozoites are released. The merozoites infect adjacent epithelial cells and repeat another asexual cycle or eventually differentiate into gametocytes, initiating sexual reproduction (Plorde, 1990). Fusion of the mature male and female gametocytes leads to the formation of an oval, thick- walled oocyst that is passed in the feces. Oocysts appear in the cat's feces from 3 to 5 days after infection

with *T. gondii,* and in a typical infection millions of oocysts are released daily for 1-3 weeks (Roberts and Janovy, 1996). The asexual stage disappears from the cat's small intestine after 3 weeks and so the cat stops producing oocysts. The oocysts are immature at the time of shedding and require oxygen for sporulation; they sporulate in 1 to 5 days (Roberts and Janovy, 1996). Upon maturation, 2 sporocysts (each containing 4 sporozoites) develop within each oocyst (approximately 10-12 μm). Once mature, the resistant oocyst may remain infectious for as long as a year (Plorde, 1990).

Asexual reproduction occurs only in the intermediate host (humans, domestic and wild animals). Intermediate hosts become infected by ingesting oocysts via infected cat feces or by eating raw or undercooked meat that contains tissue cysts. When the intermediate host swallows mature oocysts, digestive enzymes in the gastrointestinal tract disrupt the cyst wall (Plorde, 1990). Sporozoites are released from the disrupted cyst and are distributed to all parts of the body. They enter various kinds of cells, such as striated muscles and retinal cells, but ultimately the parasite tends to localize in the central nervous system (Roberts and Janovy, 1996). Following rapid asexual proliferation, the host cells rupture and liberate trophozoites known as tachyzoites. The tachyzoites are small banana-shaped crescents that measure 4-7 μm long and that must enter new host cells to initiate another asexual cycle (Bogitsh and Chang, 1990). As the number of tachyzoites increases due to repeated cycles of proliferation, the host reacts by producing specific antibodies that curtail proliferation (Dailey, 1996). When this stage is reached, the infection passes into a chronic stage characterized by the formation of intracellular cysts packed with numerous trophozoites designated as bradyzoites. (Dailey, 1996). The cysts become surrounded by a tough wall and are called tissue cysts. Eventually, tissue

cysts measure up to 200 μm in diameter and may contain more than a thousand bradyzoites. Cysts remain in the infected host for months or even years after infection, which is usually permanent.

Sporulated oocysts, tachyzoites, and tissue cysts all serve as infective agents. Feral and domestic cats will continue to be a source of infection to humans. Any cat, no matter how well fed and protected, may be passing oocysts of *T. gondii*. Particularly at risk are individuals such as children at play, who may come in close contact with areas likely to be contaminated with cat feces (i.e., sand boxes, garden soil), and adults responsible for changing "kitty litter". Ripe oocysts from fecal contamination of the environment by cats provide infection for grass-eating animals (i.e., cow, sheep). This form of the parasite easily withstands environmental extremes; oocysts can survive freezing and thawing conditions, allowing them to remain viable for years (Hays, 1996). In addition to the oral route of infection, congenital infection may occur if a woman acquires acute toxoplasmosis while she is pregnant. Furthermore, insects such as filth flies and cockroaches can mechanically transfer oocysts to human food (Roberts and Janovy, 1996). Infections via tissue cysts occur when raw or undercooked meat is eaten. House cats can be infected this way when they eat rodents and birds. Humans can be infected by ingesting undercooked meat products. Although beef is certainly a potential source of infection, pork and lamb are much more likely to be contaminated (Roberts and Janovy, 1996).

Immunity and Pathogenesis

In the primary infection, the proliferation of trophozoites results in the death of involved host cells and the stimulation of a mononuclear inflammatory reaction (Plorde,

1990). The most common symptoms of acute toxoplasmosis are painful, swollen lymph glands, fever, headache, muscle pain, anemia, and sometimes lung complications. This syndrome can be mistaken easily for the flu. In immunodeficient hosts, rapid proliferation of the parasites continues, producing numerous and widespread foci of tissue necrosis (Plorde, 1990). The consequences are most serious in organs such as the brain, where the potential for cell regeneration is limited.

In normal hosts, *T. gondii* infection is controlled by a strong cell-mediated immune response that shuts down replication of the tachyzoite stage and leads to the formation of dormant cysts (Roberts and Janovy, 1996). Cell-mediated immunity is thought to be the major host factor preventing reactivation of chronic infection as well as determining acquired resistance to reinfection (Gazzenelli et al., 1991). Both $CD4^+$ and in particular $CD8^+$ T-cell responses are involved in the resolution of infection (Khan et al., 1994). Natural killer (NK) cells also appear to play a critical role during *T. gondii* infection by producing interferon gamma (IFN-γ), which in turn activates macrophages into a microbicidal state. Studies have implicated IFN-γ as the major cytokine which plays a protective role during acute infection and prevents reactivation of latent infection (Araujo, 1992). Because *T. gondii* elicits a strong Th_1 cytokine response, interleukin-2 (IL-2) and interleukin 12 (IL-12) also appear to be principal mediators against acute parasitic infection. IL-2 is required for priming and maintaining the cell-mediated host response, and IL-12 is an essential cytokine that stimulates the proliferation of NK cells and $CD8^+$ T cells (Araujo, 1992). Chronic infection results when immunity builds up sufficiently to depress tachyzoite proliferation and coincides with the formation of tissue cysts. These cysts can remain intact for years and produce no obvious clinical effect.

Occasionally, a cyst wall will break down, releasing bradyzoites. Most of these are killed by host reactions, although some may form new cysts (Roberts and Janovy, 1996). Death of the bradyzoites may elicit an intense hypersensitive inflammatory reaction, which in the brain is gradually replaced by nodules of glial cells. If many such nodules are formed, the host may develop symptoms of chronic encephalitis. Chronic active or relapsing infections of retinal cells by tachyzoites causes blind spots and extensive infections of the central macular area, which may lead to blindness (Roberts and Janovy, 1996). Cysts and subsequent cyst rupture in the retina also can lead to blindness. Other kinds of extensive pathological conditions such as myocarditis, with permanent heart damage and with pneumonia, can occur in chronic toxoplasmosis (Roberts and Janovy, 1996).

When an infected person becomes immunosuppressed, such as in AIDS, tissue cysts rupture and a rapid dissemination of the parasite occurs. In AIDS, toxoplasmosis is thought to originate primarily from reactivation of a chronic infection. This process results in excessive cellular destruction producing mass lesions in the CNS, which may lead to encephalitis and eventually death (Gazzinelli et al., 1991). *Toxoplasmosis* is currently recognized as a serious opportunistic infections in AIDS patients (Heitman and Irizarry, 1997). It has been estimated that as many as 1 in 10 AIDS patients in the United States dies of toxoplasmosis (Hays, 1996). Thus, infection with *T. gondii* can be devastating for individuals with AIDS. In these patients, the major immunodeficiencies are a severe depletion of $CD4^+$ T lymphocytes and an impaired capacity to produce

IFN-γ. In addition, any long-term steroid therapy, such as is given to some cancer patients, can result in disseminated toxoplasmosis producing ocular toxoplasmosis and fatal CNS disorders (Roberts and Janovy, 1996).

Another tragic form of this disease is congenital toxoplasmosis. If a mother contracts acute toxoplasmosis at the time of her child's conception or during pregnancy, the parasites often will infect her developing fetus. One out of every 500 pregnant women acquires acute toxoplasmosis, and in half of those cases, the infection does spread to the fetus (Plorde, 1990). Depending on the stage when the fetus is infected, the end result could be an abortion, a still born child, or a child born with some form of mental retardation. Of those infected approximately 60% are subclinical, 9% die, and 30% suffer severe damage such as hydrocephalus, retinochoroiditis, and mental retardation (Roberts and Janovy, 1996). Toxoplasmosis is considered a major cause of human birth defects, probably causing more congenital abnormalities in the United States than rubella, herpes, and syphilis combined (Roberts and Janovy, 1996).

The Effects of Antioxidants on Immune Function

Nutrition has a profound effect on immunity and overall health in animals (Chew, 1995). Nutritional deficiencies impair responsiveness and, thereby, increase morbidity and mortality (Chew, 1995). On the other hand, nutritional supplementation often enhances certain aspects of immune function (Chew 1995). A group of vitamins (i.e., vitamin E and vitamin C) and minerals (i.e., Se and zinc) have recently received a great deal of attention because of their apparent effect on immunity and disease etiology (Chew, 1995).

The immune system protects the host from invasion and damage from a wide range of microorganisms (parasites, bacteria, molds, yeasts, fungi and viruses) by a highly complex biological response (Grimble, 1997). The response involves cellular proliferation, enhanced protein synthesis and inflammatory mediator production, and widespread physiological changes (Grimble, 1997). Although the immune system has a protective role for the host, some of the actions of the system have the potential to damage the host. Highly reactive oxygen species such as the superoxide anion radical (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) are continuously produced in the course of normal aerobic cellular metabolism (Chew, 1995). In addition, phagocytic granulocytes undergo respiratory burst to produce oxygen radicals to destroy intracellular pathogens. These activated oxygen species (AOS) can damage genetic material, inactivate membrane-bound enzymes, and cause lipid peroxidation (Florence, 1995). Lipid peroxidation in cell membranes destroys critical cellular components and affects membrane fluidity. Loss of membrane fluidity in lymphocytes has been directly related to decreased ability of lymphocytes to respond to immunological challenges (Garewal, 1997). Other sources of free radicals include strenuous exercise, detoxification, exposure to certain chemicals, radiation, ultraviolet light, alcohol, cigarette smoke, air pollutants, and high fat diets (Garewal, 1997). If these oxidative products are not eliminated, they can damage healthy cells, and thus, decrease immune function. Antioxidants serve to stabilize these highly reactive free radicals, thereby maintaining the structural and functional integrity of cells (Chew, 1995). Therefore, antioxidants are very important to the immune defense and health of animals (Chew, 1995).

Many degenerative diseases such as cancer, inflammatory joint disease, asthma, diabetes, degenerative eye disease, and atherosclerosis are believed to be caused, or at least exacerbated, by activated oxygen species (AOS) (Florence, 1995). For instance, in carcinogenesis, the initial mutagenic event often involves an AOS or some oxidative chemical (the "ultimate" carcinogen) produced by the cytochrome P-450 system (Florence, 1995). Metastasized cancer cells cannot attach to tissue without a concomitant oxidation process. There is a large body of evidence that oxidative processes, especially lipid-peroxidation, are involved in the pathogenesis of atherosclerosis (Florence, 1995). Atherosclerosis involves the deposition of fatty deposits (atheroma) in blood vessels. Atheroma; however, cannot form unless the endothelium is first damaged by AOS from white blood cells (i.e, macrophages) and lipid peroxides (Florence, 1995). Considering the involvement of activated oxygen species (AOS) in many degenerative diseases, it is not surprising that epidemiological and clinical studies have found that dietary antioxidants have a protective effect (Florence, 1995). For instance, several studies have indicated that a high intake of antioxidants leads to a decreased cardiovascular disease risk (Poppel et al., 1994). There also is evidence for an association between low vitamin intake and subsequent risk of developing some forms of cancer (Madhavi et al., 1995). The course of several other diseases, such as arthritis, can be altered by nutritional intervention (Kelley and Bendich, 1996).

Evidence has accumulated suggesting that patients infected with the human immunodeficiency virus (HIV) are under chronic oxidative stress (Pace et al., 1995). Changes in components of the antioxidant defense system and increased levels of oxidation products have been observed in asymptomatic HIV—infected patients and in

patients with acquired immunodeficiency syndrome (AIDS) (Pace et al., 1995). The role of oxidative stress in HIV disease appears to be quite broad and may involve alterations in viral replication, immune function, apoptosis, weight loss, and critical aspects of redox and glutathione-dependent metabolism (Pace et al. 1995). Furthermore, opportunistic infections, which induce a sequel of inflammatory responses, directly or indirectly, promote oxidative stress. These pro-oxidative conditions cause activation of free radical-producing immune cells, enhancement of viral replication, and weakening of the antioxidant defense system (Pace et al., 1995). Thus, nutrition plays an important role in the overall outcome of HIV-infection. Mega doses and multiple combinations of vitamins and minerals have been commonly used in an attempt to interrupt or delay progression of the disease because many vitamins have immune-modulating properties (antibody production, lymphocyte proliferation, and cytokine production) and lower free radical burden (Kelley and Bendich, 1996). Epidemiological research on the relationship between dietary nutrient intake and the progression of HIV to AIDS suggests that increased intake of vitamins (both from food and supplements) is associated with a significantly decreased progression rate to AIDS (Harbige, 1996).

Humans are well endowed with antioxidant defenses against activated oxygen species. These antioxidants, or free radical scavengers, include the enzymes catalase, superoxide dismutase, and the Se-dependent glutathione peroxidase (GSHPx). However, the antioxidant defenses of the body do not offer complete protection for the host tissues against the oxidative molecules produced by the immune system (Grimble, 1997). The capacity of the host to improve antioxidant defenses will depend in part upon the concomitant intake of nutrients such as tocopherols (vitamin E), ascorbic acid (vitamin

C) and β-carotene (Grimble, 1997). In addition, the intake of metallic micronutrients, such as Se, influences the activity of antioxidant enzymes such as the Se-dependent glutathione peroxidase (GSHpx), which further improve antioxidant defenses.

Vitamin E

Vitamin E, an essential fat-soluble vitamin, includes eight naturally occurring compounds in two classes designated as tocopherols and tocotrienols with different biological activities (Meydani, 1995). Of the eight compounds that have been isolated from plant sources that have vitamin E activity, d-α-tocopherol has the greatest biological activity and is the most widely available form of vitamin E in food (Weber et al., 1997). In mammalian cells, α-tocopherol is mainly located in mitochondrial fractions and in the endoplasmic reticulum, whereas little is found in the cytosol and peroxisomes (Chew, 1995). Vitamin E is usually expressed in international units (IU), and 1 mg of the natural form of d-α-tocopherol has a biopotency of vitamin E equal to 1.49 IU (Meydani, 1995). The recommended dietary allowance (RDA) of vitamin E for male adults is 10 mg (15 IU) per day and for female adults is 8 mg (12 IU) (Weber et al., 1997). Relative to other fat-soluble vitamins, vitamin E is safe; high doses of vitamin E provide essentially no toxicity (Meydani, 1995). Vegetables and seed oils including soybean, safflower and corn, sunflower seeds, nuts, whole grains, and wheat germ are the main sources of the tocopherols (Meydani, 1995).

The most widely accepted biological function of vitamin E is its antioxidant property (Meydani, 1995). Vitamin E, functioning as an antioxidant and free radical scavenger, can inhibit lipid peroxidation and decrease the burden of free radicals on immune cells by both quenching and reacting with oxygen radicals (Wang and Watson,

1994). During its action as a chain-breaking antioxidant in membranes, vitamin E is consumed and converted to the radical vitamin E, which in turn could be restored to vitamin E by vitamin C or other antioxidants (Wang and Watson, 1994).

Excessive alcohol (ETOH) consumption has been associated with a lowered resistance of the host to infectious diseases (Wang et al., 1994b). Alcohol consumption significantly stimulates lipid peroxidation and free radicals. Wang et al. (1994b) demonstrated that vitamin E supplementation significantly normalized lipid peroxidation and the generation of free radicals in ETOH-fed rats (Wang et al., 1994b). Furthermore, vitamin E supplementation significantly improved T- and B-cell proliferation, and slightly improved immunoglobulin production, which had been significantly reduced by dietary ETOH (Wang et al., 1994b). In addition to its antioxidant property, vitamin E may have other important roles in biological processes such as the stimulation of the immune response, the suppression of inflammation, and DNA synthesis (Dutta-Roy et al., 1994). Thus, vitamin E supplementation may improve the host defense against opportunistic infections by enhancing the immune response, which often is suppressed by ETOH ingestion (Wang et al., 1994b).

Several animal studies have shown that vitamin E is essential for normal function of the immune system. Deficiency in vitamin E is associated with a decline of the immune response, whereas higher than required amounts have stimulatory effects (Meydani, 1995). Dietary supplementation with vitamin E has been associated with increased lympho-proliferative response to mitogens, T-helper (Th) activity, antibody synthesis, neutrophil antimicrobial activity, and macrophage phagocytic activity (Wang and Watson, 1994). Chew et al. (1995) demonstrated that calves supplemented with 125 to

500 mg of α-tocopherol/d had increased T-cell and B-cell mitogenesis. Bovine peripheral blood mononuclear cells incubated *in vitro* with 55-110 μg of α-tocopherol had enhanced production of IgM and upregulation of IL-1 production (Chew, 1995). In other studies, increased vitamin E intake in mice (10 times the recommended diet level for 50 days) was associated with increased antibody responses (particularly IgG), T-helper cell function, and enhanced cooperative effects between T and B cells. Furthermore, vitamin E administered to mice intraperitoneally enhanced the mitogenic response to B-cell and T-cell mitogens, and this was achieved at an estimated 10 to 50 times the normal dietary intake (Harbige, 1996).

Animal studies have indicated that supplementation of the diet with vitamin E, at levels which are several fold greater than recommended daily requirements, increased the resistance to a number of infectious organisms (Grimble, 1997). For example, vitamin E protected chicks against experimental *Escherichia coli* infection at doses 3 and 6 times the normal dietary intake (Harbige, 1996). Following this discovery, increased resistance to infection following supplementation with vitamin E was recorded for *Histomonas meleacridis* in chicks and turkeys, *Chlamydia* in sheep and lambs, *E. coli* and *Treponema hyodysenteriae* in pigs, and *Diplococcus pneumoniae* and *Mycoplasma pulmonis* in rodents. A similar phenomenon also may occur in humans since epidemiological evidence shows lower incidence of infectious disease in subjects with high plasma tocopherol concentrations (Grimble, 1997).

Considerable evidence indicates that aging is associated with altered regulation of and decline in the immune response, which results in increased incidences of infectious diseases (Meydani, 1995). Supplementation of healthy subjects who were at least 60

years old with 800 mg d-α-tocopherol daily for 30 days increased three indices of T-cell-mediated function for which age-associated decreases have been reported (i.e., the delayed-type hypersensitivity skin test, the mitogenic response to concanavalin A, and Il-2 production (Meydani, 1995). This immunostimulatory effect of vitamin E supplementation in elderly subjects is associated with an increased resistance to disease (Meydani, 1995). In a double-blind placebo-controlled study, an enhancement of the immune response also was observed in healthy young subjects after 6 months' supplementation with 400 IU daily vitamin E (Meydani, 1995). In other studies, humans receiving 150 IU of vitamin E for 28 days showed a 50% increase in T-cell numbers (Harbige, 1996).

Acquired immune deficiency syndrome (AIDS) is a disease of presumed retroviral etiology, characterized by immune dysfunction and associated with opportunistic infections and eventual death (Wang et al., 1994a). Profound weight loss, cachexia, multiple nutrient deficiencies and protein calorie malnutrition are frequently nutritional disorders found in AIDS patients (Wang et al., 1994a). Patients at various stages of HIV infection have low serum vitamin E concentrations (Bogden et al., 1990). High intake of vitamin E was shown to enhance the immune response and reduce the size and number of carcinogen-induced tumors, which had been increased in mice with murine AIDS (Wang et al., 1994a). In addition, increased dietary vitamin E restored tissue concentrations of vitamin A, vitamin E, zinc and copper, which had been suppressed by murine AIDS. Vitamin E supplementation also partially restored secretion of important cytokines such as IL-2 and IFN-γ. Interleukin-2 and IFN-γ have distinct biological activities such as activating phagocytosis by macrophages and neutrophils, and increasing natural killer

(NK) cell activity (Wang et al., 1994a). Furthermore, vitamin E treatment of infected mice reduced levels of IL-4, IL-5, IL-6 and TNF-α, which were elevated by retroviral infection in non-supplemented mice (Wang et al., 1994a). These results suggest a possible role for vitamin E as a therapeutic nutrient to help ameliorate undernutrition and immune dysfunction caused by retroviral infection in AIDS patients (Wang et al., 1994a).

Several experimental and epidemiological studies have suggested that vitamin E might reduce the risk of cancer. A population-based control study of oral and pharyngeal cancer conducted in the United States indicated that the use of vitamin E supplements (100 IU vitamin E per day for 1 to 2 years) was associated with a 50% risk reduction for oral and pharyngeal cancer (Weber et al., 1997). In the Iowa Women's Health Study, investigators reported that use of vitamin E supplements containing more than 100 IU was associated with a highly significant reduction in the risk of colon cancer (Weber et al., 1997). It has been suggested that vitamin E inhibits mutagenesis and cell transformation primarily through its antioxidant function, eliminating oxygen free radicals and decreasing DNA damage (Meydani, 1995).

Selenium

Selenium is an essential dietary micronutrient required for normal growth, development and for the prevention of such deficiency diseases as liver necrosis and muscular dystrophy (Spallholz et al., 1973). Dietary sources of Se include meats (kidney and liver), seafood (swordfish and tuna), and vegetables such as cabbage, celery, and radish (Madhavi et al., 1995). Selenium is present in organic form in these sources, which is the major nutritional source of Se in animals and humans (Madhavi et al., 1995).

Selenium functions as an antioxidant at the cellular level. Selenium is incorporated into a number of functionally active selenoproteins, including the enzyme glutathione peroxidase (GPx- which functions (in conjunction with vitamins A, C, E) as a cellular protector against oxidative damage. Glutathione peroxidase is a key enzyme involved in the reduction of several organic hydroperoxides (ROOH) and hydrogen peroxide (H_2O_2) (Gamain et al., 1995). Such peroxides are a source of potentially injurious free radicals, which can cause peroxidation of polyunsaturated fatty acids in the cell membrane. Selenium-GPx is localized in the cytosol and mitochondria of most cells with erythrocytes and hepatocytes having particularly large amounts (Dworkin, 1994). The activity of glutathione peroxidase is closely related to the dietary intake of Se above the normal nutritional range (Levander, 1992).

A number of studies have documented that Se affects the immune functions of a host *in vivo* and that Se deficiency and supplementation correlate, respectively, with a decreased or an increased resistance of a host to challenge with foreign antigens (Kiremidjian-Schumacher et al., 1994). For instance, recent animal studies by Beck et al. (1994) have shown that mice fed on a Se-deficient diet developed inflamed hearts within 1 week of being exposed to a benign coxsackie virus (a family of viruses also thought to be responsible for Keshan disease). No such observations were made in the control group of mice fed on a normal diet. These results demonstrate that host Se status was important in determining the outcome of viral infection with the coxsackie virus. Selenium deficiency also has been shown to decrease the resistance of animals to a number of other pathogenic infections. For example, when Se-deficient and Se supplemented mice were given intravenous injections of *Candida albicans*, deaths of the

Se-deficient animals started after 2.5-3.5 d compared with 7-8.5 d in the Se-supplemented animals (Boyne and Arthur, 1986). Significantly more microorganisms were found in the kidney, liver, and spleen of the Se-deficient mice compared with the same organs of Se-supplemented animals. Selenium deficiency also was demonstrated to impair the ability of mouse neutrophils to kill *C. albicans* in *in vitro* tests (Boyne and Arthur, 1986). Thus, it was concluded that Se-deficient mice were more susceptible to *Candida albicans* infection. Further studies have indicated that Se-deficient mice were more susceptible to *Diplococcus pneumonia* Type 1 infection, and increased mortality was observed in Se-deficient rats after intraperitonel injection of *Staphylococcus aureus* (Boyne and Arthur, 1986).

Studies have indicated that Se supplementation is beneficial in improving the immune response in humans and mice. For instance, increased dietary Se has been shown to enhance immunity during microbial infection (Davis et al., 1998). The beneficial effect of Se was observed in mice infected with the protozoan parasite, *Trypanosoma cruzi*. Mice receiving 4 or 8 ppm Se (sodium selenate) administered in their drinking water exhibited the highest overall survival of 60% as compared to 0% survival in the non-supplemented groups (Davis et al., 1998). Also, the Se supplemented groups had reduced parasitemias when compared to the non-supplemented groups. Selenium also has been shown to alter the expression of the high affinity interleukin 2 receptor (IL-2-R) and its subunits, cell proliferation, and clonal expansion of cytotoxic T-lymphocytes in mice (Roy et al., 1994). Spallholz et al. (1973) demonstrated that dietary supplementation with Se significantly increased anti-sheep red blood cell (SRBC) IgM and IgG antibody titers in mice. In a further study, Spallholz et al. (1975) demonstrated

that Se administered to mice intraperitoneally (5 μg) enhanced the primary immune response to the sheep red blood cell antigen. Enhancement of the primary immune response was greatest when Se was administered prior to or simultaneously with the antigen (Spallholz et al., 1975). Other studies indicated that supplementation of lambs and ewes with Se at high non-physiological levels enhance IgG responses to a variety of infectious challenges (Harbige, 1996).

In humans, the immuno-enhancing effects of Se require supplementation above levels acquired by normal dietary intake. Dietary supplementation with Se in humans (200μg/day for 8 weeks) resulted in a 118% increase in cytotoxic lymphocyte-mediated tumor cytotoxicity and an 82.3% increase in natural killer cell activity as compared to normal baseline values (Kiremidjian-Schumacher et al., 1994). Further studies indicated that dietary supplementation with Se in humans above levels acquired by normal dietary intake resulted in a significant augmentation of the ability of peripheral blood lymphocytes to respond to mitogen (Roy et al., 1994).

Selenium deficiency is common in HIV positive patients as documented by low plasma and red blood cell levels of Se and diminished activity of glutathione peroxidase (Dworkin, 1994). Patients with AIDS tend to have more severe deficits than those with earlier stages of HIV infection. Both gastrointestinal malabsorption and deficient dietary intake in AIDS patients undoubtedly contribute to the development of Se deficiency. It has been suggested that in patients with AIDS, that Se deficiency may be associated with impaired immune function, reduced T-cell counts, as well as various specific disorders such as oral candidiasis (Dworkin, 1994). Selenium supplementation in HIV-infected patients has been shown to cause symptomatic improvements, especially in appetite and

intestinal functions, and to possibly slow the course of the disease (Garewal, 1997). Selenium supplementation during AIDS also has been shown to improve cardiac dysfunction (Dworkin, 1994). For example, Keshan disease (a myocardiopathy associated with children) is seen in low-Se regions of China and can be prevented by Se supplementation (Foster and Sumar, 1997). This similar form of cardiomyopathy also has been seen frequently in AIDS patients. Therefore, it has been suggested that Se supplementation during the course of HIV infection should be a form of supportive therapy (Garewal, 1997).

Selenium also is emerging as a dietary factor that may prove to be of major significance as a prophylactic agent against cancer (Madhavi et al., 1996). Selenium has been found to be capable of inhibiting and or retarding tumorigenesis in a variety of experimental animals. For example, Medina et al. (1983) demonstrated that Se was anticarcinogenic in mice both *in vivo* and *in vitro*. Dietary supplementation of mice with 2.0 ppm of Se markedly inhibited tumor production in mice, and this level of Se was not detrimental to the host (Medina et al., 1983). Moreover, high levels of Se given in the drinking water protected against UV-light –induced skin cancer in hairless mice (Levander, 1987). The protective effect of Se against free-radical injury is primarily attributed to the selenoenzyme GSH-Px.

The Combined Effects of Vitamin E and Se

Vitamin E has a complex relationship with Se (Chang et al., 1994). The two exert similar antioxidant effects in cells, but via independent biochemical pathways and in different locations (Chang et al., 1994). Vitamin E acts on the biomembrane and protects lipid membranes, whereas Se acts in the form of Se-dependent glutathione peroxidase

(GPX) in the cytoplasm and protects –SH groups in membrane proteins against oxidation (Zhu et al., 1992). Furthermore, vitamin E prevents lipid peroxidation more effectively than Se, whereas Se prevents free radical production more effectively than vitamin E (Zhu et al., 1992). Thus, while each can have a sparing influence on the need for the other, neither can fully compensate for the other (Chang et al., 1994).

The results of several investigations have supported the conclusion that dietary deficiencies of vitamin E and/or Se are associated with an impairment of the immune response (Chang et al. 1994). There is evidence that in the chicken, dietary deficiencies of these nutrients (either individually or together) can impair humoral immunity, cell-mediated mechanisms, mitogenic responsiveness, primary lymphoid organ development, and disease resistance (Chang et al., 1994). A combined dietary deficiency of vitamin E and Se was shown to result in a consistent depression of splenocyte proliferation in response to the T cell mitogens ConA and to the B cell mitogen, lipopolysaccharide (Chang et al., 1994). The proliferative response could be fully reconstituted only after vitamin E and Se supplementation. Dietary deficiencies also resulted in a decreased proportion of peripheral T cells and significantly decreased the number of $CD4^+$ peripheral blood leukocytes (PBL) (Chang et al., 1994). Another known physiological consequence of α-tocopherol or Se deficiency is reduced neutrophil activity (Hogan et al., 1992). Both antioxidants protect neutrophils from the destructive action of toxic oxygen molecules necessary for intracellular kill of ingested pathogens. Dietary supplementation of cattle with Se results in a more rapid neutrophil influx into milk following intramammary bacterial challenge, and increases intracellular killing of ingested bacteria by neutrophils (Hogan et al., 1993). Dietary supplementation of early

lactation cows with vitamin E results in increased bactericidal activity by bovine blood neutrophils (Hogan et al., 1993). The supplementation of deficient animals with Se and/or vitamin E improves the function of neutrophils in other animals, such as in chickens and sheep (Finch and Turner, 1996). The ability of phagocytes to migrate to the site of infection or inflammation may also be affected by an animal's Se/vitamin E status. Selenium deficiency reduced the random migration and chemotactic responses of caprine neutrophils, and both responses could be reversed by Se supplementation (Finch and Turner, 1996).

Vitamin E and Se both have been shown to have immunostimulatory effects in a variety of species when administered in quantities in excess of established dietary requirements (Sheffy and Schultz, 1979). Wise and Tomasso (1993) reported that fingerling channel catfish fed diets deficient in vitamin E were more susceptible to peroxidation than fish fed diets that met or exceeded vitamin E recommendations. Hepatic Se-dependent glutathione peroxidase activity was suppressed in fish fed diets deficient in Se when compared to fish fed diets containing recommended or higher levels of Se (Wise and Tomasso, 1993). Intracellular superoxide anion production by macrophages (an indicator of disease resistance) was higher in fish fed the diet fortified with four times the recommended levels of both nutrients than in fish fed the other diets (Wise and Tomasso, 1993).

Many of the studies of the effects of Se and vitamin E on immune responses have investigated their influence on antibody production. Studies in rodents have revealed a synergistic effect when both nutrients were administered (Finch and Turner, 1996). Combined supplementation with Se and vitamin E has been particularly effective in

raising the antibody responses of animals (chickens, pigs, and horses) previously deficient in both nutrients (Finch and Turner, 1996). The immune responses of dogs can be influenced by vitamin E and Se nutrition. Neutralizing antibody titers following vaccination with a canine distemper-infectious hepatitis virus vaccine were lower during vitamin E and Se deficiency at each period tested for a 28-day-period post-immunization (Sheffy and Schultz, 1979). The appearance of measurable antibody after immunization was delayed in vitamin E-Se deficient dogs, and the secondary immune responses also were reduced in the deficient dogs (Sheffy and Schultz, 1979).

Free radical catalyzed lipid peroxidation is a continual biological process causing damage to cellular and intracellular structures (Sheffy and Schultz, 1979). This process can act to destroy certain enzymes and other intracellular components, as well as to alter membrane structures. Vitamin E alone and in concert with Se can inhibit these processes. It has been demonstrated that the oxidative damage in many tissues is more severe during double Se and vitamin E deficiency than with deficiency of either antioxidant alone (Ndiweni and Finch, 1995).

Studies have shown that Se and vitamin E status also can have a profound impact on the ability of a host to resist acute infectious disease (Levander et al., 1995). A low vitamin E/Se status has been associated with increased vulnerability of dairy cattle to mastitis (Ndiweni and Finch, 1995). Since polymorphonuclear leucocytes (PMN) provide the major cellular defense mechanism within the mammary gland, the effect of *in vitro* supplementation with vitamin E and Se on the function of these cells was investigated. Both vitamin E and Se enhanced the functions of mammary gland macrophages and peripheral lymphocytes (Ndiweni and Finch, 1996). Furthermore, a

reduction in the incidence of subclinical infections also was associated with a high vitamin E/Se status (Ndiweni and Finch, 1996).

INTRODUCTION

The protozoan parasite *Toxoplasma gondii* is common in nature and highly prevalent in humans throughout the world (Araujo, 1992). Approximately 50% of the human population of the United States has been infected (Plorde, 1990). After acute infection with *T. gondii*, the parasite forms cysts in multiple tissues and organs, which persist for years and probably for the entire life span of the individual (Huskinson-Mark et al., 1991). Individuals with a normal functional immune system are able to contain infection by *T. gondii* but immunocompromised patients are at risk of developing life-threatening toxoplasmosis. Thus, toxoplasmosis, particularly toxoplasmic encephalitis, has emerged as a major cause of morbidity and mortality in patients with the acquired immunodeficiency syndrome (AIDS) (Suzuki and Joh, 1994). When the host is immunocompromised, as in AIDS or during immunosuppressive therapy, cysts rupture and a rapid dissemination of the parasite occurs (Gazzinelli et al., 1991). This process results in excessive cellular destruction, producing lesions in many organs, and may result in pneumonia, encephalitis, and death (Gazzinelli et al., 1991).

Since immunocompetent individuals do not suffer apparent untoward effects from their infection with *T. gondii*, it is clear that the immune response is critical for prevention of toxoplasmic encephalitis (Suzuki et al., 1996). The major mechanism of resistance to *T. gondii* is cell-mediated with the participation of $CD8^+$ and $CD4^+$ T cells (Araujo, 1992). In addition, the cytokine IFN-γ plays a crucial protective role during acute infection and in the prevention of reactivation of a latent infection (Araujo, 1992).

Humoral immunity also is known to be involved in resistance against acute toxoplasmosis (Suzuki et al., 1996).

Nutrition and nutritional status can have profound effects on immune function, resistance to infection, and autoimmunity in man and animals (Harbige, 1996). Nutrients may enhance or depress immune function depending on the nutrient and level of its intake (Harbige, 1996). Vitamin E and Se both have been shown to have immunostimulatory effects in a variety of species when administered in excess of established dietary requirements (Sheffy and Schultz, 1979). Both nutrients have been shown to influence antibody production, lymphocyte proliferation, cytokine production, and numbers of the specific subgroups of white blood cells (Kelley and Bendich, 1996).

Vitamin E is a fat-soluble vitamin which is located in cell membranes, where it protects membrane polyunsaturated fatty acids from peroxidation (Ndiweni and Finch, 1995). Studies in various animals have demonstrated that vitamin E deficiency impairs cellular and humoral immunity. Deficiency of the vitamin also is associated with an increased incidence of disease (Grimble, 1997). However, vitamin E supplementation brings about the opposite effect. Supplementation of the diet with vitamin E at levels which are several fold greater than normal results in increased resistance to a number of pathogens (Grimble, 1997). For example, resistance of chickens and turkeys to *Escherichia coli* and of mice to pneumococci was enhanced by vitamin E supplementation. Furthermore, increased intake of vitamin E has been shown to enhance interleukin-2 production and the delayed hypersensitivity reaction, lymphocyte proliferation in response to concanavalin A (Con A) and lipopolysaccharide (LPS) (Grimble, 1997).

Selenium is a co-factor of the cytosolic enzyme glutathione peroxidase (GSHPx) which is essential for the detoxification of hydrogen and lipid peroxides (Ndiweni and Finch, 1995). A number of studies have documented that Se affects the immune functions of a host and that Se deficiency and supplementation correlate, respectively, with a decreased or an increased resistance of a host to challenge with foreign antigens (Kiremidjian-Schumacher et al., 1994). Selenium supplementation studies performed with mice, lambs, and ewes indicate that at high non-physiological intake Se enhances IgG responses to a variety of infectious challenges (Harbige 1996). Selenium also has been shown to enhance the capacity of a host to generate cytotoxic lymphocytes (CTL) and macrophages and to destroy tumor cells (Kiremidjian-Schumacher et al., 1994).

Notwithstanding the demonstrated beneficial effects of vitamin E and Se supplementation on immune function, a few studies have indicated that deficiencies of vitamin E and Se can increase the resistance of animals to certain microorganisms (Boyne and Arthur, 1986). Selenium deficiency was shown to increase the survival of rats infected with *Salmonella typhimurium* and mice infected with *Plasmodium bergeii* or *Listeria monocytogenes* (Boyne and Arthur, 1986). Vitamin E deficiency protected mice against the intraerythrocytic parasite *Plasmodium yoelii* (murine malaria) by markedly improving the survival of infected mice, especially when the mice were concurrently fed peroxidizable fat such as fish or linseed oil (Levander, 1992). Recent studies have provided evidence that *Plasmodium* does possess a parasitic selenium-dependent glutathione peroxidase to protect against the deleterious effects of oxidative stress (Gamain et al., 1995). Higher glutathione peroxidase activities were observed in the malarial parasite after selenium supplementation. After Se supplementation, with either

sodium selenite or selenocystine an increase in parasitic growth was observed (Gamain et al., 1995).

Patients infected with the human immunodeficiency virus (HIV) typically experience chronic oxidative stress (Pace and Leaf, 1995). Oxidative stress is a pathologic phenomenon caused by a relative overload of oxidants, i.e., reactive oxygen species (Pace and Leaf, 1995). Reactive oxygen species (ROS) are toxic via their effects on cellular components such as the denaturation of proteins, membrane lipids, and DNA (Garewal, 1997). Any antigenic or regulatory stimulus to polymorphonuclear leukocytes, or T-cell activity, will increase the expression of ROS (Greenspan and Aruoma, 1994). Therefore, the high level of antigenic and cytokine activity in HIV/AIDS results in the production of substantial levels of superoxides, hydrogen peroxide and hydroxyl radicals. Additional sources of ROS include the products of peroxidation and sequelae of inflammatory responses induced by opportunistic infections (Greenspan and Aruoma, 1994). Thus, infection with the intracellular pathogen *T. gondii* would be expected to further exacerbate oxidative stress in HIV/AIDS individuals. As antioxidants, vitamin E and Se function as free radical scavengers and help prevent ROS from causing further cellular damage. Vitamin E and Se supplementation in HIV-infected patients has been shown to reduce free radical concentrations, lipid peroxidation and cause symptomatic improvements (Garewal, 1997). Therefore, the reduction of oxidative stress by antioxidant treatment is suggested as supportive therapy in early as well as in advanced stages of HIV infection (Garewal, 1997).

The high incidence of toxoplasmosis among AIDS patients has served as an impetus to identify new treatment strategies (Huskinson-Mark et al., 1991). The present

study was conducted to determine if vitamin E and Se might provide a beneficial effect in a murine model of chronic infection with *T. gondii*. At present, the only model for evaluation of the activity of therapeutic agents against the cyst form of *T. gondii* is the chronically infected laboratory animal (Huskinson-Mark et al., 1991). In earlier studies, vitamin E and Se were shown to provide protective activity against another intracellular parasite, *Trypanosoma cruzi* (Davis et al., 1998 and Hou, 1997). Dietary supplementation of vitamin E and Se in mice infected with *T. cruzi* resulted in increased longevity, decreased parasitemias and reduced weight loss during infection. In the present study, kinetics of *T. gondii* infection in mice were assessed by calculating percent weight loss, tissue cyst numbers and severity of tissue pathology.

MATERIALS AND METHODS

Mice

Swiss Webster mice were obtained from Hilltop Laboratories (Scottsdale, PA), and C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These two different strains of mice were chosen because Swiss Webster are not known to develop toxoplasmic encephalitis, whereas the C57BL/6J mice are more susceptible to *T. gondii* with the development of TE. All mice were females and 5 to 6 weeks of age when used. In addition, the mice were housed in groups (2 or 5 mice per group) in plastic box cages and provided rodent chow and water *ad libitum*.

Toxoplasma gondii Infection

Oocysts of the ME49 strain of *T. gondii* were used to study chronic infections in mice. The oocysts of the ME49 strain was kindly provided by Dr. David Lindsay of the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech (Blacksburg, Virginia). Prior to inoculation of intact oocysts into mice, the ME49 oocysts were washed with 10 ml of sterile DPBS (Dulbecco's Phosphate Buffered Saline; Sigma Chemical Co., St. Louis, Missouri), and the vial was then centrifuged at 1800 rpm for 15 minutes. The supernatant was removed and discarded. This washing procedure was repeated, and the pellet containing oocysts was resuspended in 5 ml of sterile of DPBS. Oocysts were counted using a hemacytometer.

After washing and counting the oocysts, an infection inoculum of 10^3 or 10^4 was administered intraperitoneally (i.p), subcutaneously (s.c) or orally to each mouse.

Experimental Design

The efficacy of vitamin E alone and in combination with Se was examined in three sets of experiments. For each antioxidant combination, a selected dose that had previously been found to be optimal in *Trypansoma cruzi*-infected mice was used (Davis et al., 1999 and Hou, 1997).

In the first phase of the study, 35 Swiss Webster mice (7 mice per group) were used to investigate the effect of dietary supplementation with antioxidants during chronic infection with *T. gondii.* Upon arrival, mice were randomly separated into 10 cages of 2 or 5 mice each and immediately placed on one of the following diets:

GROUP 1 (CONTROL)	*49 IU/kg Vitamin E and 0.2 ppm Se (Daily Recommended Allowance)*
GROUP 2 (Deficient Diet)	*Vitamin E and Se Deficient*
GROUP 3 (Se Diet)	*8 ppm Sodium Selenate*
GROUP 4 (Vitamin E Diet)	*400 IU/kg Vitamin E*
GROUP 5 (Vitamin E and Se Diet)	*400 IU/kg Vitamin E and 8 ppm Sodium Selenate*

The control diet consisted of normal laboratory rodent chow (Purina Rodent Chow #5001, Purina Mills, Richmond, IN) containing 49 IU/kg of vitamin E and 0.2 ppm Se. The deficient diet contained Purina rodent chow that had 0 IU/kg of vitamin E (#5000, Purina Mills, Richmond, IN) and 0 ppm of Se. The vitamin E diet consisted of Purina rodent chow supplemented with 400 IU/kg vitamin E (#5827, Purina Mills, Richmond, IN), and the Se diet consisted of 8 ppm of sodium selenate (Sigma Chemical Co.). Sodium selenate was administered to the relevant groups in their drinking water. The vitamin E and Se diet consisted of 400 IU/kg of vitamin E (food) and 8 ppm sodium selenate (water). Food and water were provided *ad libitum*. Mice were infected with *T.*

gondii 3 weeks after being placed on the experimental diets. All mice were inoculated subcutaneously (s.c) with 10^3 oocysts of the ME49 strain.

In the second phase of the experiment, to study chronic infections in a susceptible model with the expected development of toxoplasmic encephalitis, 25 C57BL/6J mice were divided into 5 groups (5 mice per cage). Three weeks before inoculation, the mice were fed one of the five diets described above, then all 25 C57BL/6J mice were challenged intraperitoneally (i.p) with a higher dose of oocysts (10^4) of the ME49 strain.

In the third phase of the experiment, 25 C57BL/6J mice were put under the same conditions as in the previous two experiments. However, because the natural route of *T. gondii* infection for humans and mice is the ingestion of food or water contaminated with oocysts from cat feces (Dubey et al., 1997), the C57BL/6J mice were challenged orally by directly pipetting oocysts (10^4) of the ME49 strain into the mouth.

Weight

In the first two phases of the study, mice were weighed upon arrival, on the day before they were infected with *T. gondii,* and throughout the course of infection.

Histological Evaluation

Histological studies were performed 8 weeks (chronic stage) after infection. Brains were removed intact from SW and C57BL/6J mice infected s.c or i.p with the ME49 strain of *T. gondii*. The brains were sectioned in half (along the midline). Therefore, histological studies were performed on the left side of the brain, and enumerations of tissue cysts were done on the right side of the brain.

The left side of the brain of each mouse was cut in half and the front half was placed in fixative (10% formalin). The tissue was processed and tissue sections were

obtained using routine histological techniques. Fixed tissue was placed in a series of graded ETOH baths beginning with 65%, 70%, 80%, 2 changes in 95% and 2 changes in absolute ETOH. Tissue remained in ETOH baths for 45 minutes each.

After the dehydration series, the tissue was cleared using xylene (two changes in xylene, 45 minutes each). From the clearing agent, the tissue was then prepared for embedding (embedding medium was paraffin wax, two changes in wax, 45 minutes each). Paraffin-embedded sections were then blocked and cut into 5-μm sections. The distance between sections was 50 microns. For each tissue sample, there were 2 slides, 4 sections per slide. Paraffin-embedded sections were then stained with hematoxylin and eosin, and were examined with light microscopy to evaluate the severity of tissue pathology. Brain lesions were based on the following 2 criteria: (1) mean size of foci and (2) the mean number of inflammatory foci per section. Eight sections from each mouse were examined, and a total of 24 sections were examined for each group.

Cyst Number Determination

The number of tissue cysts in the brain was determined by a technique described by Huskinson–Mark et al. (1991). The right side of the brain was placed in 1 ml of DPBS and triturated until the suspension appeared homogenous with a mortar and pestle (6 strokes). Ten μl of Tween 20 was added to the suspension. The suspension was then passed 10 times through an 18-gauge needle and 20 times through a 22-gauge needle. Two 20-μl aliquots of each sample were placed on glass microscope slides that were then mounted with coverslips. Cysts were counted microscopically by scanning each 20-μl aliquot at a magnification of 10X.

Statistical Evaluation

Data were analyzed using SYSTAT. ANOVA (Analysis of Variance) was used to test the effect of diet on mice infected with *T. gondii*. Dependent variables in the ANOVA included weight, tissue cysts, size of foci, and the number of inflammatory foci. Tissue cyst number was the only variable transformed in the first phase of the study, and was transformed using the $\log_{10}$-function. The number of tissue cysts refers to the mean number of tissue cyst present in the brains of mice after eight weeks of infection. Weight refers to the percent weight change during the initial 2 weeks of infection. The size of foci refers to the average size of foci per section per group of mice. The number of foci refers to the mean number of inflammatory foci per section per group of mice.

RESULTS

Effect of Diet on Weight

The weights of all mice were taken in experiment 1 and 2. The average weights of Swiss Webster mice prior to infection, 2 weeks post infection, and 8 weeks post infection with *T. gondii* are shown in Figure 1. Surprisingly, mice in the vitamin E plus Se group (vitamin E and Se in levels in excess of normal dietary intake) showed the greatest average weight loss during the initial 2 weeks of *T. gondii* infection. The percent weight loss for this group was more than 3 fold greater as compared to the deficient group, Se only group, and the vitamin E only group (see Table 1a; 9.57 % versus 0.65-2.97%). The control group (daily recommended levels of vitamin E plus Se) was the only group of mice that showed a percent weight gain during the initial 2 weeks of infection. However, there were no statistically significant differences in percent weight change among the different groups of Swiss Webster mice ($P > 0.05$), and by the eighth week of infection, all groups of mice had gained weight (Figure 1).

Figure 2 shows the effect of diet on weight in *T. gondii*-infected C57BL/6J mice. Mice in the vitamin E plus Se group showed the greatest percent weight loss among all the groups during the initial 2-week period of infection (see Table 1b; 6.49%). Percent weight loss in the vitamin E plus Se group was statistically significant among the other groups ($F = 6.010$, $df = 20$, $P = 0.002$). Mice on the Se only diet showed the second highest percent weight loss, followed by mice on the control diet (daily recommended levels of vitamin E plus Se), deficient diet, and vitamin E only diet (Table 1b; 4.04%,

3.17%, 2.70% and 1.01%, respectively). Between the second and eighth week of infection, all C57BL/6J mice had gained weight (Figure 2).

Effect of Diet on Tissue Cyst Number

The effect of dietary supplementation with vitamin E and Se on number of tissue cysts was determined in *T. gondii*-infected Swiss Webster and C57BL/6J mice. Table 2a shows the average number of tissue cysts in the brains of Swiss Webster mice inoculated subcutaneously with 10^3 oocysts of the ME-49 strain. Counts were performed in the eighth week of infection. Mice in the vitamin E plus Se group (vitamin E and Se in levels in excess of normal dietary intake) had the greatest number of cysts in their brains. In contrast, mice in the deficient group (absence of vitamin E and Se from the diet) had the fewest number of cysts in their brains (a mean of 64 versus a mean of 339 in the vitamin E plus Se group). Mice on the Se only diet, vitamin E only diet, and the control diet had a mean number of 117, 96, and 89 tissue cysts, respectively. Overall, the vitamin E plus Se group showed a significant statistical difference in tissue cyst number among the other groups of mice ($F = 3.14$, $df = 27$, $P = 0.030$).

Table 2b shows the number of tissue cysts in *T. gondii*-infected C57BL/6J mice inoculated intraperitoneally with 10^4 oocysts. The C57BL/6J mice are more susceptible to the ME49 strain and are known to develop toxoplasmic encephalitis during infection. As in the previous experiment, mice in the supplemented groups had the highest number of tissue cysts present when compared to the deficient group (Table 2b). However, there were no statistically significant differences between the groups of mice ($P > 0.05$).

In the third experiment, C57BL/6J mice were infected orally with 10^4 *T. gondii* oocysts. Similar to the results seen in experiment 2, the deficient group had the fewest

number of tissue cysts present (mean of 95); (see Table 2c). Among the supplemented groups, mice on the Se only diet had the highest number of cysts (mean of 235), followed by mice on the vitamin E only diet (mean of 230), vitamin E plus Se diet (190) and the control diet (mean of 165). Despite these differences, there was no statistically significant difference between the deficient group and the supplemented groups ($P > 0.05$).

Effect of Diet on Histopathology

At 8 weeks post infection, Swiss Webster and C57BL/6J mice were killed and histological analysis was performed on the left side of each brain. The histopathological findings for *T. gondii* infected Swiss Webster mice are summarized in Table 3a. Mice in all groups showed evidence of cellular infiltration. However, chronic inflammatory lesions in the brains of mice in the vitamin E plus Se group (vitamin E and Se in levels in excess of normal dietary intake) tended to be more severe (Figure 3). Mice in the vitamin E plus Se group showed more numerous and larger sites of focal inflammation in the parenchyma of the brain when compared to mice in the deficient and control group (daily recommended levels of vitamin E plus Se), (see Figure 3b, d and f). The brains from mice on the vitamin E plus Se diet also showed a remarkable infiltration of the meninges (see Figure 3e). In contrast, the number of inflammatory cells present in the meninges was notably less in the brains of the control mice and deficient mice (Figure 3a, and c). The degree of histopathology appeared to be similar in the brains of the mice in the vitamin E group (absence of Se) and Se group (absence of vitamin E); (data not shown). Mice in both supplemented groups tended to have larger inflammatory foci than mice in the deficient and control groups (Table 3a). Despite these observed differences in

histopathology, there was no statistically significant difference in mean number or mean size of inflammatory loci in the brain parenchyma of representative mice from each group.

Results of the histopathological analysis in C57BL/6J mice infected with *T. gondii* are presented in Table 3b. Similar to the previous experiment, the inflammatory response was much more remarkable in the vitamin E plus Se group than in the deficient group (Figure 4). The brains of the vitamin E plus Se group (vitamin E and Se in levels in excess of normal dietary intake) and control group showed an intense infiltration of mononuclear cells within the subarachnoid space of the meninges (Figure 4c, and e). In contrast, the deficient group showed far less inflammation in the parenchyma and very low numbers of inflammatory cells infiltrating the meninges (Figure 4a, and b). The overall results indicate that mice in the supplemented groups had more numerous areas of infiltration by mononuclear cells and larger areas of chronic focal inflammation when compared to mice in the deficient group (Table 3b). However, there were no statistically significant differences in mean size of foci ($F = 3.216$, $df = 9$, $P = 0.067$) or mean number of inflammatory foci ($F = 1.99$, $df = 9$, $P = 0.178$) among the groups of mice.

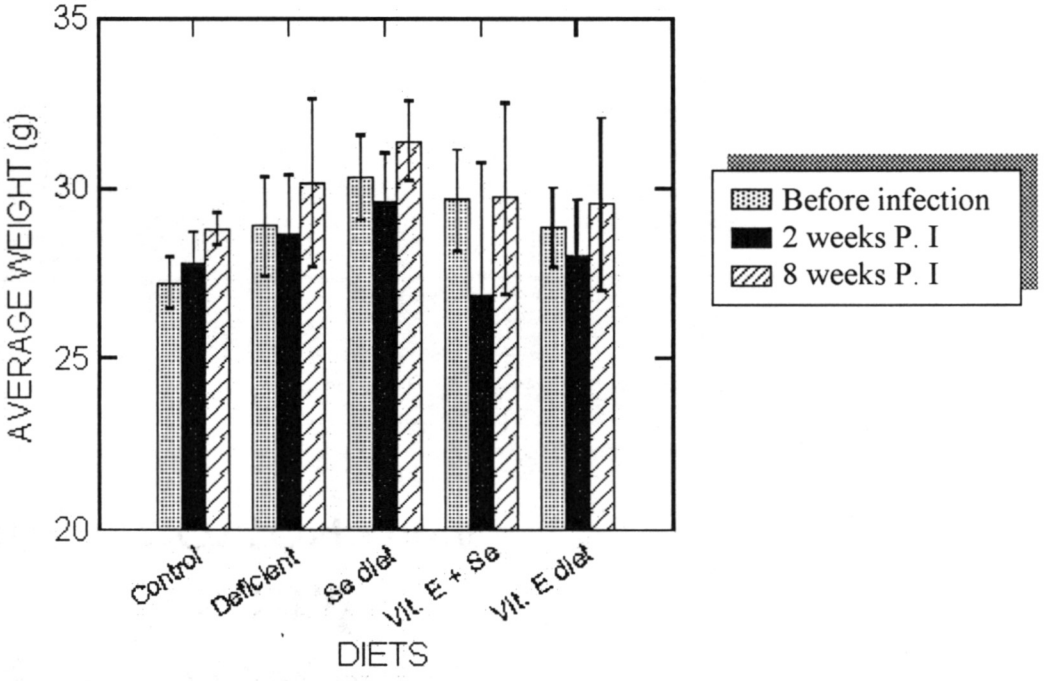

FIGURE 1. Effect of diet on weight in *T. gondii*-infected Swiss Webster mice. The average weight of individual mice in each group was calculated before infection with *T. gondii,* 2 weeks post-infection (P. I), and 8 weeks P. I. Data are presented as average weight ± standard deviation of weights of 7 mice in each group.

TABLE 1. Percent weight change in *T. gondii*-infected Swiss Webster mice (**a**) and C57BL/6J mice (**b**). The percent weight change per gram of average weight before infection with *T. gondii* to 2 weeks post-infection (P. I) were calculated for each group of mice.

a.

GROUPS	% WEIGHT CHANGE
Control Diet	-2.01
Deficient Diet	0.65
Se Diet	2.27
Vitamin E + Se Diet	9.57
Vitamin E Diet	2.97

b.

GROUPS	% WEIGHT CHANGE
Control Diet	3.17
Deficient Diet	2.70
Se Diet	4.04
Vitamin E + Se Diet	6.49
Vitamin E Diet	1.01

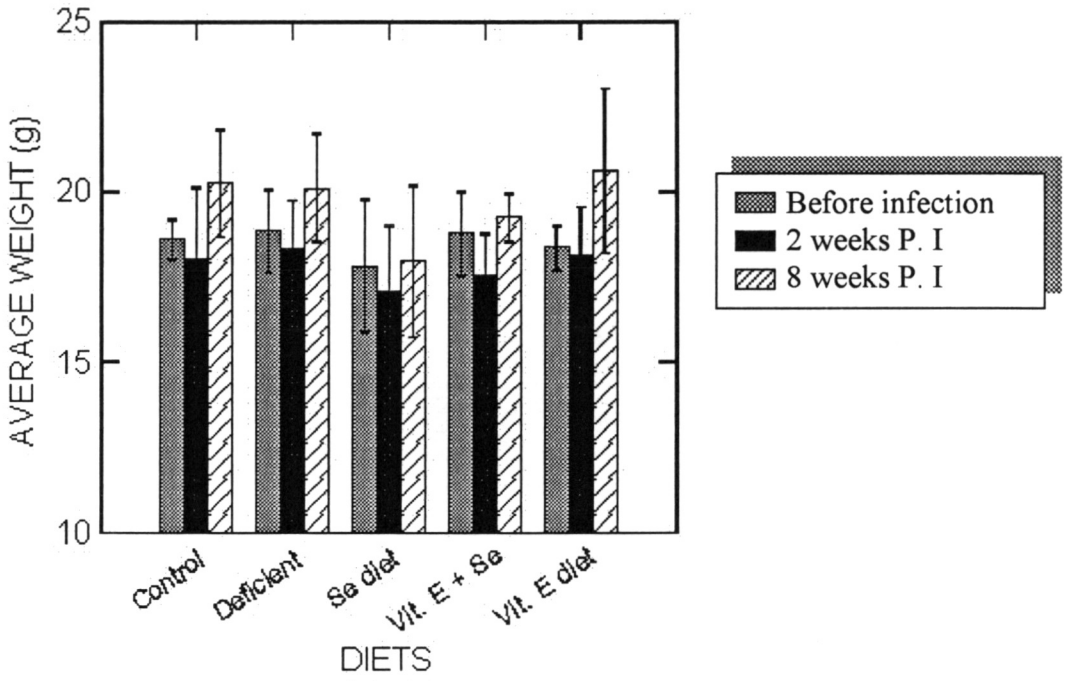

FIGURE 2. Effect of diet on weight in *T. gondii*-infected C57BL/6J mice. The average weight of individual mice in each group was calculated before infection with *T. gondii*, 2 weeks post-infection (P. I), and 8 weeks P. I. Data are presented as average weight ± standard deviation of weights of 5 mice in each group.

TABLE 2. Number of tissue cysts in *T. gondii*-infected Swiss Webster mice inoculated subcutaneously (**a**), C57BL/6J mice inoculated intraperitoneally (**b**), and C57BL/6J mice infected orally (**c**). Cysts number was determined at 8 weeks post-infection. Data are presented as the mean number of tissue cysts present in 1000 µl (1/2 of brain homogenate).

a.

GROUPS	NO. OF CYSTS
Control Diet	89
Deficient Diet	64
Se Diet	117
Vitamin E + Se Diet	339
Vitamin E Diet	96

b.

GROUPS	NO. OF CYSTS
Control Diet	180
Deficient Diet	45
Se Diet	170
Vitamin E + Se Diet	155
Vitamin E Diet	145

c.

GROUPS	NO. OF CYSTS
Control Diet	165
Deficient Diet	95
Se Diet	235
Vitamin E + Se Diet	190
Vitamin E Diet	230

TABLE 3. Effect of diet on histopathology in the brain of Swiss Webster mice (**a**) and C57BL/6J mice (**b**) chronically infected with *T. gondii.* Data are presented as the mean size (micrometers) of foci per group of mice and the average number of inflammatory foci per section per group of mice. Eight sections from each mouse were examined. A total of 24 sections were examined for each group of mice (8 sections from 3 different mice).

a.

GROUPS	MEAN SIZE OF FOCI (μm)	NO. OF INFLAMMATORY FOCI PER SECTION
Control Diet	139.15	6.38
Deficient Diet	158.25	6.13
Se Diet	173.47	5.08
Vitamin E + Se Diet	227.18	8.13
Vitamin E Diet	171.55	8.45

b.

GROUPS	MEAN SIZE OF FOCI (μm)	NO. OF INFLAMMATORY FOCI PER SECTION
Control Diet	175.97	8.39
Deficient Diet	102.50	2.50
Se Diet	164.95	8.19
Vitamin E + Se Diet	179.84	13.63
Vitamin E Diet	167.95	8.21

FIGURE 3. Histological sections from the brains of Swiss Webster mice chronically infected with *T. gondii* (ME-49) and fed the different diets. A, Brain section from a Swiss Webster deficient mouse (absence of vitamin E and Se from diet). Note the lack of inflammation in the meninges. B, Brain section from a Swiss Webster deficient mouse. No inflammation is apparent in the brain parenchyma. C, Brain section from a Swiss Webster control mouse (diet containing daily recommended levels of vitamin E and Se). The meninges show infiltration by large numbers of inflammatory cells (arrows). D, Brain section from a Swiss Webster control mouse. The brain parenchyma shows an area of chronic inflammation with cellular infiltration (arrow). E, Brain section from a Swiss Webster vitamin E plus Se mouse (diet containing vitamin E and Se in amount in excess of normal dietary intake). Note the widespread chronic inflammation present within the meninges. (arrows). F, Brain section from a Swiss Webster vitamin E plus Se mouse. The brain parenchyma shows a massive area of infiltration by inflammatory cells (arrows). Bar, 100 μm.

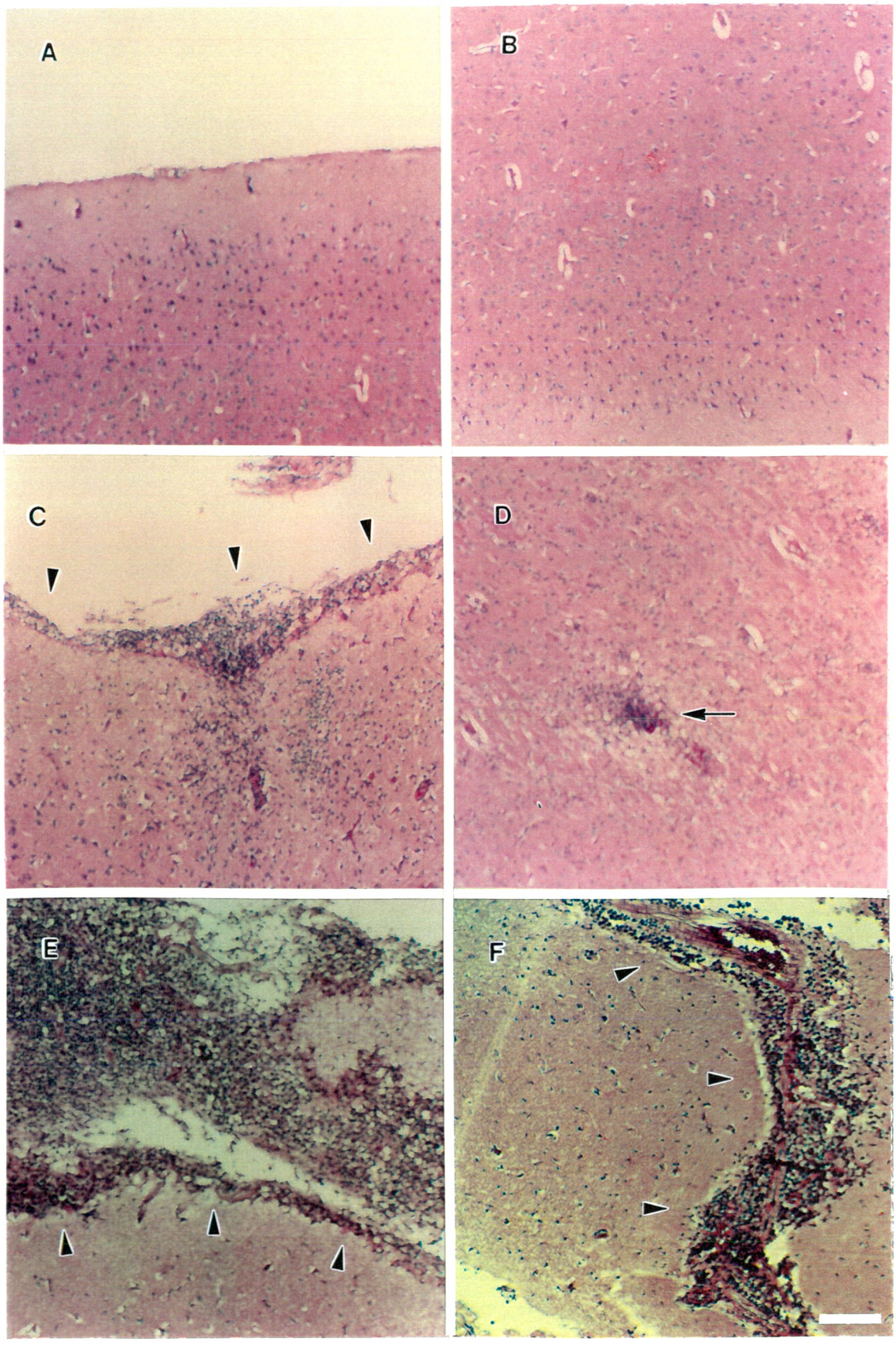
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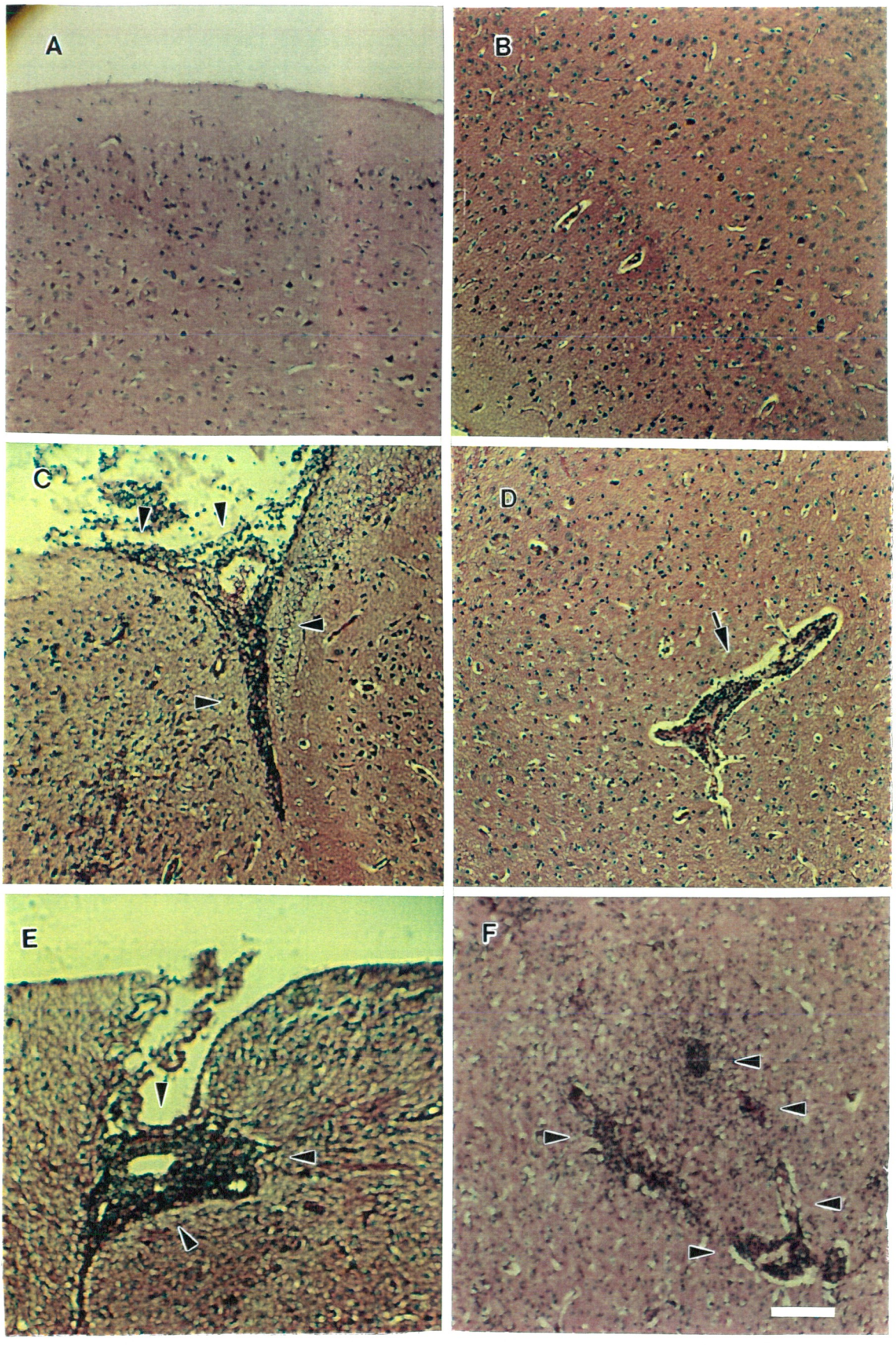
FIGURE 4. Histological sections from the brains of C57BL/6J mice chronically infected with *T. gondii* (ME-49) and fed the different diets. A, Brain section from a C57BL/6J deficient mouse (absence of vitamin E and Se from diet). Note the lack of inflammation in the meninges. B, Brain section from a C57BL/6J deficient mouse. No inflammation is apparent in the brain parenchyma. C, Brain section from a C57BL/6J control mouse (diet containing daily recommended levels of vitamin E and Se). Large numbers of inflammatory cells are seen infiltrating the subarachnoid space of the meninges (arrows). D, Brain section from a C57BL/6J control mouse. The brain parenchyma shows a large area of cellular infiltration (arrow). E, Brain section from a C57BL/6J vitamin E plus Se mouse (diet containing vitamin E and Se in amount in excess of normal dietary intake). Note regions of cellular infiltration within the meninges (arrows). F, Brain section from a C57BL/6J vitamin E plus Se mouse. The brain parenchyma shows several areas of infiltration by inflammatory cells (arrows). Bar, 100 μm.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DISCUSSION

In the present study the effects of dietary supplementation with vitamin E and Se during murine infection with *T. gondii* were investigated. The severity of infection was evaluated by monitoring weight, tissue cyst numbers, and the extent of tissue pathology.

Vitamin E and Se supplementation has been associated with an enhancement of the immune response, increasing resistance to a variety of pathogenic infections and tumors in animal models and humans (Wang et al., 1994a). Previous studies in our laboratory have shown that supplementation of mice with vitamin E and Se does provide benefit against the intracellular parasite, *Trypanosoma cruzi* (Davis et al., 1998 and Hou, 1997). Dietary supplementation with vitamin E and Se in mice infected with *T. cruzi* resulted in increased longevity, decreased parasitemias and reduced weight loss during infection. The beneficial effects of vitamin E and Se may be attributable in part to the well known antioxidant properties of both of these nutrients. Selenium is an essential dietary micronutrient required for normal growth and development, and functions as an antioxidant at the cellular level. Selenium is necessary for the synthesis and activity of the peroxide-destroying enzyme glutathione peroxidase. Glutathione peroxidase functions as a cellular protector against oxidative damage by catalyzing the reduction of hydrogen peroxide and organic hydroperoxides (Madhavi et al., 1995). Vitamin E is a lipid-soluble antioxidant that protects polyunsaturated fatty acids (PUFAs) and other components of cell and organelle membranes from oxidation by reactive free radicals (Dutta-Roy et al., 1994). Several studies have indicated that both antioxidants show a

synergistic effect in depressing lipid peroxide formation *in vivo* and *in vitro* (Madhavi et al., 1995). In addition, vitamin E and Se have been shown to have immunostimulatory effects in a variety of species when administered in quantities in excess of established dietary requirements (Sheffy and Schultz, 1979). Supplementation with vitamin E and Se has been shown to improve the function of neutrophils, and to increase antibody responses of animals (chickens, pigs, and horses) (Finch and Turner, 1996). However, the results of the present study demonstrate that vitamin E and Se supplementation was not beneficial during murine infection with the protozoan parasite, *T. gondii.* Increased dietary vitamin E and Se supplementation resulted in increased cyst number, tissue pathology, and weight loss during infection. In contrast, mice fed the deficient diet (absence of vitamin E and Se) showed the lowest number of tissue cysts and very little evidence of tissue pathology during chronic infection.

Weight was monitored throughout the study as an indicator of severity of disease. Although there were no statistically significant differences in percent weight loss in the Swiss Webster mice, the vitamin E plus Se group (vitamin E plus Se in levels in excess of normal dietary intake) showed the greatest percent weight change during the first two weeks of infection. Similar results were obtained in the second phase of the study. C57BL/6J mice on the vitamin E plus Se diet also had the highest percent weight loss during the initial 2 weeks of infection ($P < 0.005$). Percent weight loss over the first two weeks of infection is a good indicator of disease severity because this is the time when mice are showing other clinical signs of acute illness, such as ruffled coat, decreased activity, and decreased appetite. Two weeks after infection, mice pass the acute phase and go into the chronic phase of the disease which is characterized by the formation of

tissue cysts in the brain. In the present study, increased weight loss during the acute phase of infection was correlated with an increased tissue cyst burden in the chronic stage of infection. In the first experiment, the Swiss Webster mice in the vitamin E plus Se group had the greatest number of tissue cysts present ($P < 0.05$). In the second experiment, the C57BL/6J in the vitamin E plus Se group and the Se only group suffered the highest percent weight loss, and this too was correlated with a high tissue cyst burden in their brains. The C57BL/6J supplemented groups had high tissue cyst numbers compared to the C57BL/6J deficient group (Table 2). This trend was similar in the third phase of the study, in which C57BL/6J mice were inoculated orally (natural route) using oocysts as the inoculum. Mice in the supplemented groups had the greatest number of tissue cysts in their brains compared to the deficient group (Table 2). These results strongly suggest that dietary supplementation with vitamin E and Se in both mouse models of *T. gondii* infection is actually detrimental to the host, whereas a pro-oxidant diet (one totally lacking in vitamin E and Se) appears to be beneficial.

The number of cysts in the brain appears to be one of the major factors that determine development of toxoplasmic encephalitis (Suzuki et al., 1993). Therefore, it would be expected that mice in the supplemented groups would be more likely to exhibit toxoplasmic encephalitis, since they formed a greater number of tissue cysts than mice in the non-supplemented groups. This prediction is consistent with the histological evaluation of inflammatory responses in the brains of mice chronically infected with *T. gondii*. Eight weeks after *T. gondii* infection, mice in the Swiss Webster vitamin E plus Se group showed evidence of severe meningo-encephalitis with an extensive infiltration by large numbers of inflammatory cells within the subarachnoid space of the meninges.

Swiss Webster mice in the vitamin E group, Se group, and control group all had apparent inflammation; however inflammatory lesions in the vitamin E plus Se group were remarkably more severe (particularly within the meninges) (Figure 3e). In the second phase of the histological study, C57BL/6J mice in the supplemented groups also exhibited severe menigo-encephalitis (Figure 4). The brain parenchyma of mice in the C57BL/6J vitamin E group and Se group also showed several areas of chronic focal inflammation populated by very large numbers of mononuclear cells (Table 3). Inflammatory cells also were noted within the subarachnoid space of the meninges of the brains. Despite the obvious clinical differences observed between the groups of Swiss Webster and C57BL/6J mice, there were no statistically significant differences in brain lesion size, or the number of foci. This result may be attributable to the small sample size used in the histological analysis and the large variance between individual mice within groups.

In both Swiss Webster and C57BL/6J mice, the lowest degree of tissue pathology and cyst number was exhibited by mice in the deficient group (absence of vitamin E and Se). In striking contrast, results of a variety of studies have shown that supplementation with antioxidants can provide a beneficial effect during microbial infection (Davis et al., 1998, Grimble, 1997, and Hou, 1997). However, similar results have been reported in other studies. Selenium deficiency was shown to increase survival of rats infected with *Salmonella typhimurium* and mice infected with *Plasmodium bergeii*, *Listeria monocytogenes*, or *Pseudorabies* virus (Boyne and Arthur, 1986). In a study involving mice infected with the helminth parasite, *Schistosoma mansoni*, it was shown that a diet deficient in vitamin E and Se impaired the development of *S. mansoni* by decreasing the

number of adult worms and liver surface nodule scores (Levander et al., 1995). Levander et al. (1995) also have reviewed the effects of vitamin E supplementation during malaria. Malaria is a disease caused by an intraerythrocytic parasite belonging to the genus *Plasmodium*. *Plasmodium* lives in an oxygen rich environment (the red blood cell) and is known to be susceptible to the negative effects of oxidative stress. When mice were fed dietary fish oil (a known tocopherol antagonist), it partially protected the mice against murine cerebral malaria. However, that protection could be made virtually complete when the fish oil was fed to vitamin E-deficient mice. In addition, vitamin E deficiency markedly improved the survival of infected mice fed on fish oil, whereas all the mice died in the vitamin E supplemented group (Levander et al., 1995). Levander et al. (1995) showed that a variety of fish oils and fish-oil concentrates fed in the diet exerted this antimalarial activity in vitamin E-deficient mice. A plant oil containing *n*-3 fatty acids (linseed oil) also had this antimalarial property in vitamin-E deficient mice as did ground flaxseed and chemically purified ethyl linolenate (Levander et al., 1995). The biochemical mechanism by which fish oils exert their beneficial effect against malaria is not certain since these oils are known to have a wide-ranging impact on several aspects of metabolism. Nonetheless, the most reasonable and straightforward explanation for this antimalarial property of fish oil seems to be its ability to exert pro-oxidant stress on the parasite (Levander et al., 1995). The malarial parasite is highly sensitive to oxidative stress and a pro-oxidant diet should promote the accumulation of oxygen free radicals (i.e., superoxide anion, singlet oxygen, hydroxyl radicals, and hydrogen peroxide), thereby increasing oxidative stress and causing the destruction of the malarial parasite. Dietary induced oxidative stress provided protection against the later sequelae of malarial

infection (i.e., anemia) by suppressing parasite growth, but also protected against the early manifestations of the disease (central nervous system consequences; headaches, convulsions and coma) (Levander et al., 1995). Thus, dietary deprivation of vitamin E in this experimental model was beneficial to the host in its defense against the malarial parasite. In a similar study of mice infected with *Babesia rodhani* (Levander et al., 1995), it was demonstrated that mice fed fish oil in a low vitamin E diet were protected from the pathological effects of *B. rodhani*. Similarly, young chicks maintained on flaxseed oil (pro-oxidant diet) exhibited decreased severity of caecal lesions when infected with the coccidial parasite, *Eimeria tenella* (Levander et al., 1995). Gamain et al. (1995) have studied the effects of selenium supplementation during the *in vitro* cultivation of *Plasmodium*. They observed an increase in the growth of *Plasmodium falciparum* (human malaria) and *Plasmodium yoeli* (murine malaria) in cultures supplemented with sodium selenite. The authors concluded from the study that the malarial parasite might possess an endogenous Se-dependent enzyme, such as glutathione peroxidase (Se-Gpx), to protect itself from the deleterious effects of oxidative stress (Gamain et al., 1995). Glutathione peroxidase is a key enzyme involved in the reduction of several organic hydroperoxides (ROOH) and hydrogen peroxide (H_2O_2). Selenium is a metal cofactor for Se-GPx, and Se must be incorporated in order for this enzyme to be expressed. Based upon the results of these studies, it was proposed that *Plasmodium* might be using the antioxidants vitamin E and Se for its own benefit, for protection from oxidative stress, as well as promotion of parasitic growth. *Babesia*, *Eimeria*, *Plasmodium* and *Toxoplasma* all belong to the phylum Apicomplexa, and these organisms may use a common mechanism in their defense against oxidative damage.

The present study strongly suggests that dietary supplementation with vitamin E and Se does increase the severity of murine infection with *T. gondii* as shown by an increase in cyst numbers, tissue pathology and percent weight loss, when compared to mice maintained on a pro-oxidant diet totally lacking in vitamin E and Se. Furthermore, Swiss Webster mice are not noted for developing toxoplasmic encephalitis during *T. gondii* infection, whereas C57BL/6J mice are regarded as a more susceptible model of infection and also serve as a common model for toxoplasmic encephalitis. However, the results of the present study indicate that Swiss Webster mice receiving a lower inoculum actually exhibited more severe pathology than C57BL/6J mice. Overall, Swiss Webster mice on the vitamin E plus Se diet had the highest number of tissue cysts present in their brains and the most severe meningo-encephalitis. The number of cysts in the brain appears to be one of the primary factors that determine development of toxoplasmic encephalitis (Suzuki et al., 1993). This result suggests that Swiss Webster mice (who do not normally develop toxoplasmic encephalitis) can be rendered susceptible to toxoplasmic encephalitis if maintained on a diet containing high levels of the antioxidants vitamin E and Se.

The results of this study may have important implications for patients infected with HIV and who also are infected with *T. gondii*. Most cases of toxoplasmosis in HIV patients occur during the late stage of AIDS (Harbige, 1996). Nutrition in relation to HIV/AIDS has attracted the attention of scientists and people with HIV/AIDS for many reasons (Harbige, 1996). Vitamin and mineral deficiencies are common among both symptomatic and asymptomatic HIV-infected individuals, and nutritional supplementation in an attempt to correct these deficiencies is common (Spach et al,

1996). In addition, megadoses and multiple combinations of vitamins and minerals have been used commonly in an attempt to interrupt or delay progression of disease (Spach et al., 1996). However, in the case of an AIDS patient, suffering from toxoplasmosis, would these vitamin and mineral supplements still be beneficial? Toxoplasmic encephalitis (TE) remains a major cause of morbidity and mortality in patients with AIDS (Suzuki et al., 1993). If supplementation with antioxidants favors the growth of *T. gondii* rather than providing benefit to the host, then dietary supplementation with certain vitamins and minerals with antioxidant properties could actually exacerbate the infection and lead to serious complications in immunocompromised individuals. Further studies must be performed to fully evaluate the impact of dietary supplementation with antioxidants during *T. gondii* infection in mice as well as in humans.

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